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Iron Deficiency and Erythropoietin Excess: Two Sides of the Same Coin?

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Effect of Erythropoietin on Fibroblast Growth Factor 23 in Mice and Humans

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ABSTRACT

Background

Erythropoietin (EPO) has been reported as a novel determinant of fibroblast growth factor 23 (FGF23) production; however, it is unknown whether FGF23 is stimulated by chronic exposure to EPO or by EPO administration in non-polycystic chronic kidney disease (CKD) models.

Methods

We analyzed the effects of chronic EPO on FGF23 in murine models with chronically high EPO levels and normal kidney function. We studied the effects of exogenous EPO on FGF23 in wild type mice, with and without CKD, injected with EPO. Also, in four independent human CKD cohorts, we evaluated associations between FGF23 and serum EPO levels or exogenous EPO dose.

Results

Mice with high endogenous EPO have elevated circulating total FGF23, increased disproportionately to intact FGF23, suggesting coupling of increased FGF23 production with increased proteolytic cleavage. Similarly, in wild type mice with and without CKD, a single exogenous EPO dose acutely increases circulating total FGF23 out of proportion to intact FGF23. In these murine models, the bone marrow is shown to be a novel source of EPO-stimulated FGF23 production. In humans, serum EPO levels and rhEPO dose are positively and independently associated with total FGF23 levels across the spectrum of CKD and after kidney transplantation. In our largest cohort of 680 renal transplant recipients, serum EPO levels are associated with total FGF23, but not intact FGF23, consistent with the effects of EPO on FGF23 production and metabolism observed in our murine models.

Conclusion

EPO affects FGF23 production and metabolism, which may have important implications for EPO-treated CKD patients.

INTRODUCTION

The development of anemia[1] and elevated fibroblast growth factor 23 (FGF23) levels[2] are among the earliest changes observed in chronic kidney disease (CKD), and complex relationships among FGF23, anemia, and anemia-related factors are emerging. It has recently been demonstrated that iron deficiency[3-6] and erythropoietin[7-10] are previously unrecognized non-mineral determinants of FGF23 production. Characterization of the relationships among these factors is important as, in CKD, both elevated FGF23 levels and anemia are associated with disease progression,[11-15] cardiovascular morbidity,[16-20] and all-cause mortality.[12,21,22] Furthermore, higher EPO dosing is itself associated with cardiovascular morbidity and mortality in CKD.[23,24]

A critically important hormone in CKD-mineral bone disorder (CKD-MBD), FGF23 is secreted by osteocytes, induces phosphaturia, and decreases renal 1α-hydroxylase expression,[25] physiologically functioning as a homeostatic regulator of phosphate and a counterregulatory hormone to 1,25-dihydroxyvitamin D. FGF23 levels increase early in the course of CKD and continue to rise as the glomerular filtration rate decreases.[2,26-28] Regulation of FGF23 in CKD remains incompletely understood. Several factors may increase FGF23 production, including phosphate,[29,30] 1,25-dihydroxyvitamin D,[29,31] parathyroid hormone (PTH),[32,33] calcium,[34] inflammation,[5] and iron deficiency.[3-6]

In murine models demonstrating the effect of iron deficiency on FGF23, the iron deficiency is accompanied by anemia, raising the question of whether other anemia-related factors, such as EPO, may also affect FGF23 metabolism. Indeed, recombinant human EPO (rhEPO) acutely increases circulating FGF23 levels in rodents with normal kidney function,[7-10] mice with CKD (*Jck* model of polycystic kidney disease),[7] and humans with normal kidney function.[7] The effect of EPO on FGF23 production may be relevant for patients with impaired kidney function, both during early and late stages of CKD. Early on, endogenous serum EPO levels increase as hemoglobin declines.[35] As CKD progresses and increased serum EPO concentrations become insufficient to maintain adequate hemoglobin levels, exogenous rhEPO is administered to bolster erythropoiesis.

In order to further investigate links between EPO and FGF23, we characterized murine models with high endogenous EPO levels; assessed the effects of a single rhEPO dose in a (non-polycystic) murine CKD model; and evaluated associations between endogenous serum EPO levels and FGF23 in non-dialysis, pre- and post-transplant human CKD cohorts, and between exogenous rhEPO dose and FGF23 in dialysis patients.

Concise methods

Full methods are detailed in the **Supplemental Material**. To assess the effects of endogenous EPO on FGF23, we characterized murine models with chronically high EPO levels: transgenic mice overexpressing human EPO (Tg6 mice, which develop relative

iron deficiency), transgenic EPO mice supplemented with iron, and beta thalassemia intermedia mice (*Hbb*$^{th3/+}$ mice, which are iron loaded). To assess the effects of exogenous EPO on FGF23, we injected wild type C57BL/6 mice, with and without 0.2% adenine diet-induced CKD, with a single intraperitoneal rhEPO dose (~67 units/gram) and assessed FGF23 parameters 6 and 24 hours post-injection. At the time of euthanasia, we collected whole blood, plasma, serum, livers, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes. We assessed bone *Fgf23* mRNA expression, marrow *Fgf23* mRNA expression, plasma C-terminal (total) FGF23, and plasma intact FGF23 (iFGF23), among other parameters. Whereas the C-terminal (total) FGF23 assay detects both intact FGF23 and C-terminal FGF23 fragments, thus functioning as a surrogate measure of all translated FGF23, the intact FGF23 assay detects only full-length, bioactive FGF23.

To assess associations between serum EPO levels or rhEPO dose and FGF23 levels in humans with CKD, we characterized multiple human cohorts across the spectrum of CKD, including pre-dialysis, dialysis-dependent, and post-kidney transplant CKD patients. The non-transplant CKD patients were from the University of California Los Angeles (UCLA). The post-transplant CKD patients were from the University Medical Center Groningen (UMCG). The post-transplant patients comprised the largest cohort, numbering 680 subjects. Multiple linear regression models were developed to investigate EPO-FGF23 associations. Models were adjusted for age, sex, estimated glomerular filtration rate (GFR), time since transplantation (in the post-transplant cohort), calcium, phosphate, parathyroid hormone (PTH), hemoglobin, ferritin, and C-reactive protein (CRP) levels. Mediation analysis was performed to assess whether hemoglobin mediated the association between EPO and FGF23 independent of adjustment for potential confounders.

RESULTS

Characterization of transgenic EPO-overexpressing mice

To assess the effects of chronically high endogenous EPO levels on FGF23, we characterized 7 to 11-week-old transgenic mice overexpressing human EPO (Tg6 mice[36]). Whereas the wild type littermates had undetectable plasma human EPO, the Tg6 mice had increased human EPO concentrations (mean 295 ± 69 mIU/mL). Compared to their wild type littermates, the Tg6 mice were polycythemic (**Fig. 1a**), had similar kidney function (**Fig. 1b**), had similar phosphate levels (**Fig. 1c**), and were relatively iron deficient, as indicated by significantly lower liver iron and hepcidin (**Figs. 1e-f**). The Tg6 mice had evidence of increased FGF23 production, with significantly elevated bone *Fgf23* mRNA expression, marrow *Fgf23* mRNA expression, and circulating total FGF23 levels (mean (SD): 3175 (1271) vs. 340 (40) pg/ml, $p<0.001$) (**Figs. 1g-i**). Circulating intact FGF23 levels

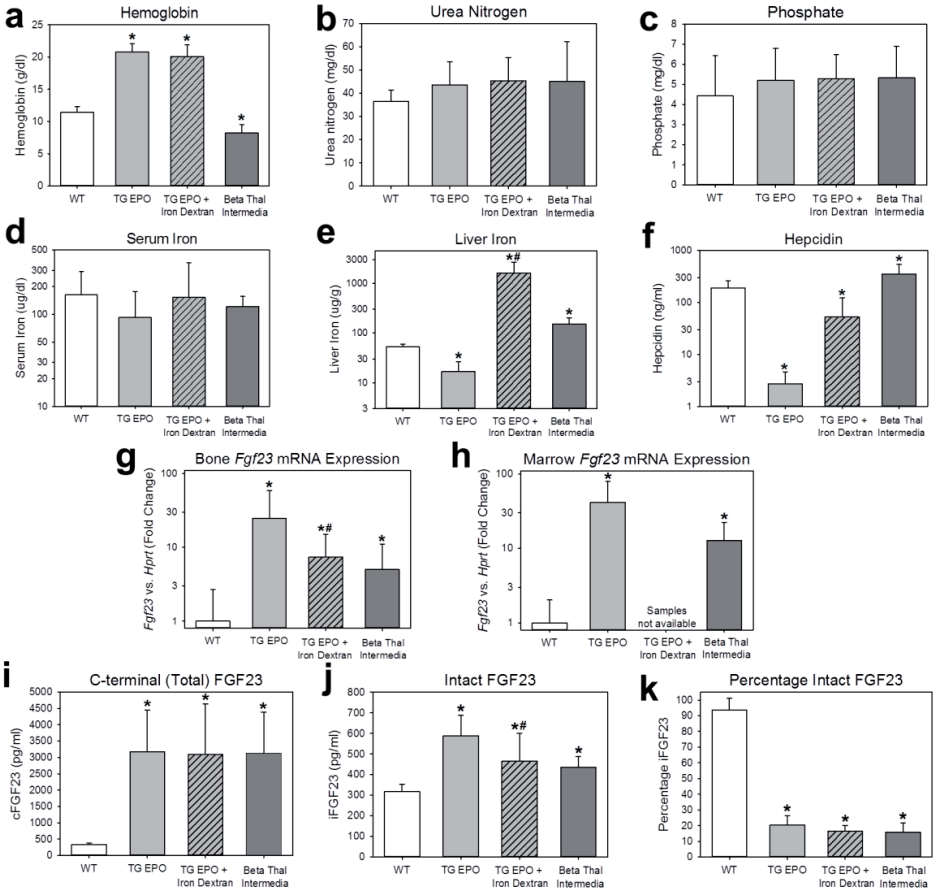

Figure 1. Characterization of FGF23 in transgenic erythropoietin overexpressing mice and beta thalassemia intermedia mice. Groups included are wild type mice (WT), transgenic erythropoietin overexpressing mice (TG EPO), transgenic erythropoietin overexpressing mice treated with a single intraperitoneal dose of 10 mg iron dextran 24 hours prior to euthanasia (TG EPO + Iron Dextran), and beta thalassemia intermedia mice. Parameters were measured at 7-11 weeks of age for the WT and TG EPO mice, and at 9-28 weeks of age for the beta thalassemia intermedia mice, and include (a) hemoglobin, (b) serum urea nitrogen, (c) serum phosphate, (d) serum iron, (e) liver iron, (f) hepcidin, (g) bone *Fgf23* mRNA expression, (h) marrow *Fgf23* mRNA expression, (i) plasma C-terminal (total) FGF23, (j) plasma intact FGF23, and (k) percentage intact FGF23. * denotes a statistically significant pairwise comparison versus the WT group ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). # denotes a statistically significant pairwise comparison of TG EPO versus TG EPO + Iron Dextran ($p<0.05$). Data are presented as means and standard deviations. n = 7-12 mice per group.

were also significantly increased (589 (100) vs. 317 (36) pg/ml, p<0.001; **Fig. 1j**), but to a much lesser extent than total FGF23 levels. Indeed, the percentage of circulating FGF23 that was intact was very low (**Fig. 1k**), suggesting that, in this model, increased FGF23 production is coupled, albeit incompletely, with increased proteolytic cleavage of FGF23.

The Tg6 mice were relatively iron deficient, which may increase FGF23 levels.[3-6] Iron dextran treatment of Tg6 mice improved iron parameters (**Figs. 1d-f**). However, iron dextran treatment only partially decreased bone *Fgf23* mRNA expression (**Fig. 1g**), did not change circulating total FGF23 levels (**Fig. 1i**), and only partially decreased circulating iFGF23 levels (**Fig. 1j**). Therefore, high EPO levels, as opposed to relative iron deficiency, predominantly drive increased FGF23 production in this model.

Characterization of beta thalassemia intermedia mice

To assess the effects of chronically high endogenous EPO levels on FGF23 in a contrasting murine model, we characterized beta thalassemia intermedia mice (*Th3*/+). Like the Tg6 mice, the *Th3*/+ mice had high EPO levels (mean serum mouse EPO 1690 ± 620 pg/ml), but they differed in their hemoglobin and iron status. Whereas the Tg6 mice were polycythemic and relatively iron deficient, the *Th3*/+ mice were anemic and iron loaded (**Figs. 1a,d-f**). Despite differences in iron status, FGF23 production was similarly elevated in the two models, correlating with high endogenous EPO levels. The *Th3*/+ mice had significantly increased bone *Fgf23* mRNA expression, marrow *Fgf23* mRNA expression, and circulating total FGF23 levels (3129 (1256) vs. 340 (40) pg/ml, p<0.001) (**Figs. 1g-i**). Circulating intact FGF23 levels were also significantly increased (436 (52) vs. 317 (36) pg/ml, p<0.001; **Fig. 1j**), but to a much lesser extent than total FGF23 levels, again suggesting coupling of increased FGF23 production with increased FGF23 proteolytic cleavage.

Administration of rhEPO to mice with normal and impaired kidney function

To evaluate the effects of exogenous EPO administration on FGF23, we analyzed wild type mice, with and without CKD, 6h and 24h after a single intraperitoneal injection of rhEPO. Urea nitrogen concentrations in the EPO-treated non-CKD and CKD groups did not differ from baseline or compared to saline-treated time point controls (**Fig. 2a**). In the EPO-treated non-CKD and CKD groups, serum phosphate did not differ from baseline or relative to saline-treated time point controls (**Fig. 2b**). Serum iron decreased only at the 24h time point in the EPO-treated non-CKD group, and did not differ from baseline in the EPO-treated CKD group (**Fig. 2c**). In the EPO-treated non-CKD and CKD groups, at the 6h time point, there were large increases in bone *Fgf23* mRNA (**Fig. 2d**), marrow *Fgf23* mRNA (**Fig. 2e**), and circulating total FGF23 levels vs. baseline (non-CKD: geometric mean (95% confidence interval) of 3289 (2305, 4692) vs. 207 (182, 236) pg/ml, p<0.001; CKD: 9376 (2280, 38,559) vs. 2056 (1282, 3297) pg/ml, p = 0.007; **Fig. 2f**), dem-

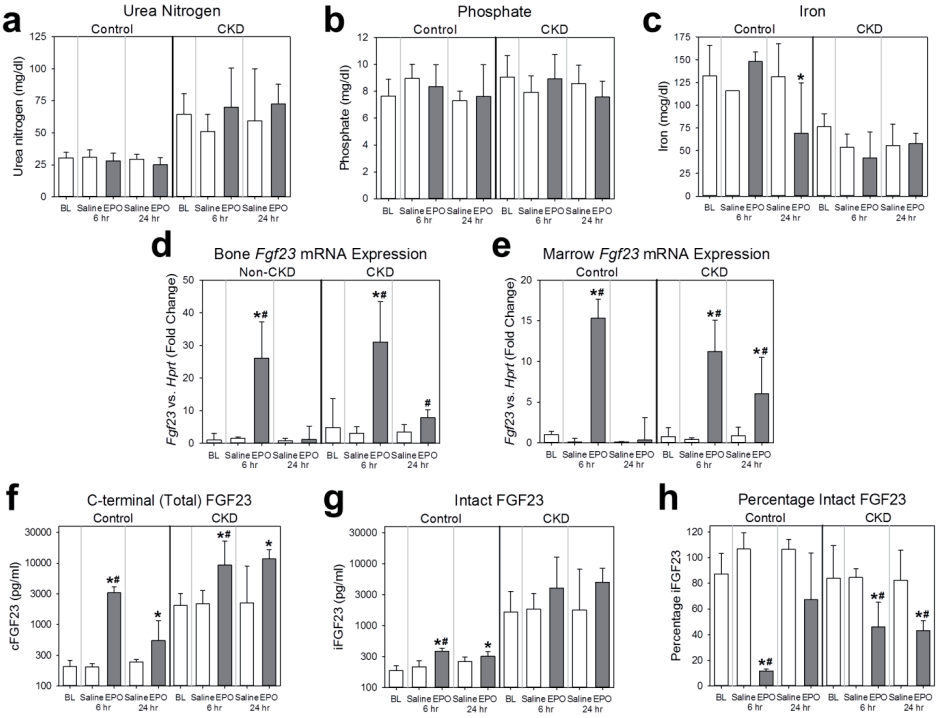

Figure 2. Acute effects of rhEPO on FGF23 in wild type mice with and without CKD. Groups included are wild type mice, with and without adenine diet-induced CKD; intraperitoneally injected with a single dose of ~67 units/gram rhEPO or saline vehicle; at baseline (BL), 6 hours post-injection, and 24 hours post-injection. Parameters shown are (a) serum urea nitrogen, (b) serum phosphate, (c) serum iron, (d) bone *Fgf23* mRNA expression, (e) marrow *Fgf23* mRNA expression, (f) plasma C-terminal (total) FGF23, (g) plasma intact FGF23, and (h) percentage intact FGF23. * denotes a statistically significant pairwise comparison of an EPO-treated group versus baseline ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). # denotes a statistically significant pairwise comparison of an EPO-treated group versus the saline-treated group at the same time point ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). Data are presented as means and standard deviations. n = 4-6 mice per group, with the exception of the baseline non-CKD group, which contained 8-20 mice, depending on the parameter measured.

onstrating EPO-induced increased FGF23 production. In the non-CKD group, circulating intact FGF23 levels also significantly increased at the 6h time point (385 (322, 461) vs. 187 (171, 203) pg/ml, $p<0.001$; **Fig. 2g**), but to a lesser extent than total FGF23 levels. In the CKD group, changes in intact FGF23 did not reach statistical significance (4046 (653, 25,079) vs. 1648 (741, 3665) pg/ml, $p = 0.17$). The calculated percentage intact FGF23 in the EPO-treated CKD and non-CKD groups at the 6h time point was decreased (**Fig. 2h**), consistent with a coupling of FGF23 production with proteolytic cleavage. At the 24h time point, the effect of EPO on FGF23 parameters was diminished, more so in the non-CKD group than in the CKD group.

Expression of enzymes involved in FGF23 cleavage

In our mice with high endogenous EPO levels, and in our mice injected with rhEPO, we assessed bone and marrow mRNA expression of enzymes involved in FGF23 cleavage, including N-acetylgalactosaminyltransferase 3 (GALNT3), FAM20C, Furin, and PC5/6 (Pcsk5).[37,38] In our high endogenous EPO models, we observed significantly decreased bone and marrow *Galnt3* mRNA expression, no difference in *Fam20c* mRNA expression, no difference in *Furin* mRNA expression, and significantly decreased bone and marrow *Pcsk5* mRNA expression (**Supplemental Figure 1**). In our CKD and non-CKD mice injected with rhEPO, at 6h post-injection, we observed no significant differences in bone or marrow *Galnt3*, *Fam20c*, or *Furin* mRNA expression (**Supplemental Figure 2**). We observed no changes in bone *Pcsk5* mRNA expression; however, marrow *Pcsk5* mRNA expression was significantly decreased in the EPO-treated non-CKD mice (**Supplemental Figure 2**).

Associations between serum EPO and circulating FGF23 levels in non-dialysis CKD patients

We next assessed associations between serum EPO and circulating FGF23 levels in a cohort of non-dialysis CKD patients. Characteristics of this cohort are listed in **Supplemental Table 1**. This cohort of 42 CKD patients included both adult and pediatric subjects. The mean eGFR was 34±18 ml/min/1.73m^2, and no patient received rhEPO. In multiple linear regression modeling, after adjusting for age, eGFR, calcium, phosphate, PTH, TSAT, ferritin, hemoglobin, and CRP, serum EPO was positively associated with Log cFGF23 ($\beta = 0.48$, $p = 0.001$; model adjusted $R^2 = 0.51$). However, the association was attenuated when Log iFGF23 was substituted for Log cFGF23 in the fully adjusted model as the dependent variable (EPO $\beta = 0.28$, $p = 0.07$; model adjusted $R^2 = 0.41$). Qualitatively similar results were obtained when iFGF23 was measured with the Kainos assay (EPO $\beta = 0.27$, $p = 0.06$). In the fully adjusted model, Log cFGF23 was not associated with hemoglobin ($p = 0.22$) and, in a model adjusted for the aforementioned covariables, hemoglobin was not associated with serum EPO ($p = 0.35$).

Associations between rhEPO dose and circulating FGF23 levels in dialysis patients

We next assessed associations between rhEPO dose and circulating FGF23 levels in a cohort of dialysis patients. Characteristics of this cohort are listed in **Supplemental Table 1**. This cohort of 79 dialysis patients included both adult and pediatric subjects. In multiple linear regression modeling, after adjusting for age, calcium, phosphate, PTH, TSAT, ferritin, hemoglobin, and CRP, rhEPO/kg was positively associated with Log cFGF23 ($\beta = 0.22$, $p = 0.033$; model adjusted $R^2 = 0.43$). However, the association was attenuated when Log iFGF23 was substituted into the fully adjusted model as the dependent variable (rhEPO/kg $\beta = 0.13$, $p = 0.21$; model adjusted $R^2 = 0.41$). In a model adjusted

for Log age, Log cFGF23, calcium, phosphate, Log PTH, TSAT, Log ferritin, and Log CRP, hemoglobin was inversely associated with Log rhEPO/kg (β = -0.24, p = 0.038). Log cFGF23 was not associated with hemoglobin (p = 0.54) in the previously described fully adjusted model.

Associations between serum EPO and circulating FGF23 levels in kidney transplant recipients

We next assessed associations between serum EPO and circulating FGF23 levels in a large cohort of adult kidney transplant patients. Characteristics of this cohort are listed in **Table 1**. In this cohort of 680 stable kidney transplant recipients, the mean age was 53±13 years, the mean eGFR was 52±19 ml/min/1.73m^2, and assessment occurred at a median of 5.4 (1.9 – 12.1) years post-transplant. In univariable analysis, Log EPO was associated with Log cFGF23 levels (β = 0.24, p <0.001), but not Log iFGF23 levels (β = 0.04, p = 0.35). In multivariable analysis, after adjusting for age, sex, eGFR, time since transplantation, calcium, phosphate, Log PTH, hemoglobin, Log ferritin, and Log CRP, Log EPO remained positively and significantly associated with Log cFGF23 (β = 0.14, p <0.001). Further, in a multivariable stepwise backward regression analysis, Log EPO remained one of the major determinants of Log cFGF23 as shown in **Table 2**. Total FGF23 was also independently and inversely associated with hemoglobin (β = -0.20, p <0.001), and hemoglobin was independently and inversely associated with Log EPO (β = -0.16, p <0.001), both after adjustment for age, sex, eGFR, time since transplantation, serum phosphate, and Log CRP levels. Mediation analysis was performed to quantify how much of the association between total FGF23 and EPO was explained by variation in hemoglobin. In a multivariable model adjusted for age, sex, eGFR, time since transplantation, serum phosphate, and Log CRP levels, hemoglobin mediated only 8.3% of the association between Log EPO and Log cFGF23 (**Supplemental Table 2**). In a sensitivity analysis, we excluded kidney transplant patients on erythropoiesis stimulating agents (ESA, n=15), and repeated the multivariable model. Log EPO remained independently and positively associated with Log cFGF23 (β = 0.13, p <0.001).

DISCUSSION

FGF23 is a key hormone in CKD pathophysiology, contributing to the maintenance of normophosphatemia until late-stage CKD,[2,28] but likely at the expense of adverse, "off-target" effects such as CKD progression[11-13] and cardiac hypertrophy.[17] Studies from our group and others have demonstrated that EPO stimulates FGF23 production, but couples increased transcription with increased post-translational cleavage, thus attenuating effects on bioactive intact FGF23 levels.[7-10]

Table 1. Baseline characteristics of the post-transplant CKD cohort consisting of 680 RTRs.

		Tertiles of erythropoietin (IU/L)			
	All patients (n=680)	**T1 (n=226) [1.0-5.0]**	**T2 (n=226) [5.1-9.4]**	**T3 (n=228) [9.5-539]**	*P* value
Age (years)	53±13	50±13	54±13	56±12	<0.001
Male sex (n, %)	383 (56)	132 (58)	131 (58)	120 (53)	0.37
Body mass index, kg/m^2	26.6±4.8	25.8±4.2	27.1±5.1	27.1±4.9	0.006
Body surface area (m^2)	1.9±0.2	1.9±0.2	2.0±0.2	1.9±0.2	0.12
Alcohol use (n, %)	551 (81)	182 (81)	179 (79)	190 (83)	0.50
Smoking status					0.14
Never smoker (n, %)	306 (45)	110 (49)	104 (46)	92 (40)	
Former smoker (n, %)	293 (43)	85 (38)	93 (41)	112 (49)	
Current smoker (n, %)	81 (12)	31 (14)	27 (12)	23 (10)	
Time since transplantation (yrs)	5.4 (1.9-12.1)	5.1 (1.8-12.1)	5.6 (1.8-11.0)	5.8 (2.2-13.7)	0.46
Diabetes mellitus[a] (n, %)	161 (24)	37 (16)	58 (26)	66 (29)	0.005
Systolic blood pressure (mmHg)	136±17	135±17	135±18	137±17	0.51
Diastolic blood pressure (mmHg)	82±11	83±12	82±11	83±10	0.63
Laboratory measurements					
iFGF23 (pg/mL)	61 (43-100)	61 (46-103)	59 (42-92)	63 (47-106)	0.18
cFGF23 (RU/mL)	140 (95-234)	131 (91-184)	137 (94-204)	172 (107-323)	<0.001
Hemoglobin (g/dL)	13.2±1.7	13.3±1.6	13.4±1.7	13.0±1.9	0.03
MCV (fL)	91±6	91±5	91±6	90±7	0.94
Ferritin (µg/L)	118 (54-222)	141 (80-238)	115 (52-242)	92 (42-184)	<0.001
TSAT (%)	25.4±10.6	28.1±10.2	25.2±9.8	22.8±11.1	<0.001
Total cholesterol (mmol/L)	5.1±1.1	5.2±1.2	5.1±1.0	5.1±1.2	0.76
Phosphate (mmol/L)	1.0±0.2	1.0±0.2	1.0±0.2	1.0±0.2	0.98
Calcium (mmol/L)	2.40±0.15	2.42±0.15	2.39±0.16	2.40±0.14	0.24
PTH (pmol/L)	8.9 (5.9-14.8)	8.5 (5.5-13.8)	9.1 (6.2-14.5)	9.2 (6.3-16.8)	0.16
eGFR[b] (ml/min/1.73m^2)	52±19	50±19	52±19	52±19	0.33
Creatinine (µmol/L)	138±59	146±69	135±55	133±51	0.03
Proteinuria[c] (n, %)	154 (23)	51 (23)	41 (18)	62 (27)	0.07
hs-CRP (mg/L)	1.6 (0.7-4.6)	1.4 (0.6-3.1)	1.7 (0.8-4.9)	1.8 (0.8-5.3)	0.01
Treatment					
ACE-inhibitors (n, %)	220 (34)	89 (39)	72 (32)	59 (26)	0.009
Bèta-blocker (n, %)	428 (63)	138 (61)	138 (61)	152 (67)	0.38
Calcium channel blockers (n, %)	165 (24)	47 (21)	67 (30)	51 (22)	0.06
Diuretic use (n, %)	275 (40)	76 (34)	88 (39)	111 (49)	0.004
ESA use (n, %)	15 (2)	7 (3)	1 (0)	7 (3)	0.09
Iron supplements (n, %)	41 (6)	14 (6)	11 (5)	16 (7)	0.63

Values are means ± standard deviation, medians (interquartile range) or proportions (%). Abbreviations: ACE, Angiotensin Converting Enzyme; cFGF23, C-Terminal Fibroblast Growth Factor 23; CKD, chronic kidney disease; hs-CRP, high-sensitivity C-reactive protein; iFGF23, intact fibroblast growth factor 23; PTH, parathyroid hormone; RTRs, renal transplant recipients; TSAT, transferrin saturation.

[a] Diabetes was defined as the use of antidiabetic medication or a fasting plasma glucose ≥7.0 mmol/L.

[b] eGFR was determined using the MDRD equation

[c] Proteinuria was defined as urinary protein excretion ≥0.5 g/24 h

Table 2. Univariable and multivariable linear regression modeling of determinants of circulating total FGF23.

Parameter	Univariable analysis		Multivariable analysis	
	std. ß	*P* value	std. ß	*P* value
EPO (IU/L)	0.24	<0.001	0.14	<0.001
Age (years)	0.08	0.05		
Male sex (yes vs. no)	0.05	0.18	-0.09	0.01
Time since transplantation (yrs)	-0.04	0.36		
eGFR (ml/min/1.73m^2)	-0.50	<0.001	-0.38	<0.001
Calcium (mmol/L)	0.01	0.78		
Phosphate (mmol/L)	0.34	<0.001	0.20	<0.001
PTH (pmol/L)	0.17	<0.001	0.12	<0.001
Ferritin (µg/L)	-0.34	<0.001	-0.33	<0.001
Hemoglobin (g/dL)	-0.37	<0.001	-0.09	0.005
hs-CRP (mg/dL)	0.19	<0.001	0.14	<0.001

Univariable linear regression analysis follow by multivariable stepwise backward linear regression analysis. cFGF23, EPO, ferritin, hs-CRP, and PTH levels have been log transformed due to skewed distribution.
Abbreviations: eGFR, estimated glomerular filtration rate; EPO, erythropoietin; FGF23, fibroblast growth factor 23; hs-CRP, high-sensitivity C-reactive protein; PTH, parathyroid hormone.

In the current study, we extend the evidence that EPO, both endogenous and exogenous, affects FGF23 production and metabolism to the settings of chronically increased circulating EPO levels and CKD, both pre- and post-transplant. In our murine models with high endogenous EPO concentrations—the transgenic EPO overexpressing mice and the beta thalassemia mice, circulating total FGF23 levels are markedly elevated and increased out of proportion to intact FGF23 levels, demonstrating that increased FGF23 production is coupled, albeit incompletely, with increased FGF23 cleavage. Importantly, these effects are observed independent of iron status, which is another factor that affects both FGF23 production and metabolism.[3-6,22] In our wild type mice injected with a single dose of rhEPO, similar effects were observed: large increases in circulating total FGF23 levels, with much less of an effect on intact FGF23 concentrations. Important to note is that, in the CKD model, changes in intact FGF23 did not reach statistical significance. Our human data is consistent with the observations made in the murine models. Across the spectrum of CKD, serum EPO levels or rhEPO doses are independently associated with total FGF23, but not intact FGF23, suggesting a coupling of increased production with increased cleavage.

Our results provide further evidence that EPO-stimulated bone marrow represents a novel source of FGF23 production. Our high endogenous EPO murine models and our wild type mice treated with rhEPO had increased marrow *Fgf23* mRNA expression. This was not caused by contaminating bone fragments within the marrow, as the flushed

marrow samples had little *Col1a1* mRNA expression, suggesting the presence of very few residual bone cells (**Supplemental Figure 3**). We have previously demonstrated that mice with pharmacologically ablated bone marrow have attenuated increases in circulating total FGF23 levels (with no change in bone *Fgf23* mRNA expression) in response to EPO.[7] Furthermore, isolated murine bone marrow cells treated *ex vivo* with EPO demonstrate acute increases in *Fgf23* mRNA expression.[7] Although it is unknown which bone marrow cell subpopulation produces FGF23 in response to EPO, it has been shown that EPO markedly increases *Fgf23* mRNA expression in Ter119+ cells (erythroid lineage);[8] in lineage negative, Sca1 positive, c-kit positive (LSK) cells, a hematopoietic progenitor cell subset;[7] but not in common myeloid progenitor (CMP) cells.[7]

Since in our murine models, total FGF23 is increased out of proportion to intact FGF23, elevations in total FGF23 levels represent mostly increased C-terminal fragments, the biological activity of which is uncertain. Previously, it has been reported that C-terminal FGF23 may function as an FGF23 antagonist, competing with intact FGF23 for binding to the FGF receptor.[39] Additionally, it has been shown in vitro that treatment with C-terminal FGF23 increases the cell surface area of adult rat ventricular myocytes.[40] In further studies, the biological relevance of elevated concentrations of C-terminal FGF23 fragments needs to be delineated in more detail.

The mechanism by which EPO increases bone and marrow FGF23 transcription is currently unknown. Furthermore, it is unknown how EPO increases FGF23 post-translational cleavage. Regulation of FGF23 post-translational cleavage is a complex process involving several enzymes, including N-acetylgalactosaminyltransferase 3 (GALNT3), FAM20C, Furin, and PC5/6 (Pcsk5).[37,38] Furin and PC5/6 are proprotein convertases that can cleave FGF23; GALNT3 glycosylates FGF23 at its cleavage site, inhibiting proteolysis; and FAM20C phosphorylates FGF23 at its cleavage site, inhibiting GALNT3-mediated glycosylation. In the current study, we assessed bone and marrow *Galnt3*, *Fam20c*, *Furin*, and *Pcsk5* mRNA expression. In the mice with chronically elevated endogenous EPO levels, we observed decreased bone and marrow *Galnt3* mRNA expression, which may allow for increased FGF23 cleavage.[41] In these mice, there were no changes in *Fam20c* or *Furin* mRNA expression, but we did observe decreased bone and marrow *Pcsk5* mRNA expression, despite increased FGF23 cleavage. Further studies are needed to elucidate the mechanisms by which EPO may directly or indirectly affect FGF23 cleavage.

Interestingly, recent studies have also described a converse direct relationship between FGF23 and EPO. In wild type mice, the administration of recombinant human FGF23 decreases serum EPO concentrations,[42] and the administration of an FGF23 blocking peptide increases serum EPO concentrations.[43] These data suggest that FGF23 may have negative regulatory effects on erythropoiesis. In human CKD patients, elevated circulating total FGF23 levels are associated with both prevalent and incident anemia.[54] We observed a similar inverse relationship between total FGF23 and hemoglobin in our

post-transplant CKD cohort. Given the inverse association between circulating total FGF23 and hemoglobin, and the inverse association between hemoglobin and serum EPO levels,[35] the observed positive association between serum EPO and total FGF23 may be bidirectional in nature. As supported by our murine studies, EPO may directly increase total FGF23 levels. Conversely, higher total FGF23 may be indirectly associated with higher serum EPO via lower hemoglobin concentrations. However, mediation analysis in the post-transplant CKD cohort revealed that variation in hemoglobin concentrations explained relatively little of the positive association between serum EPO and total FGF23, supporting the possibility of a direct effect of EPO to increase total FGF23 levels.

In summary, the current results demonstrate that FGF23 production and cleavage are increased irrespective of iron status in murine models with high endogenous EPO concentrations and in wild type mice with and without CKD treated with a single rhEPO dose. In addition, we have shown in human studies across the spectrum of CKD and post-renal transplantation that serum EPO and rhEPO dose, independently of iron status and hemoglobin, are positively associated with circulating total FGF23 levels, but not intact FGF23. Again, this suggests that EPO leads to an upregulated production and concomitantly increased cleavage of FGF23. Further studies are needed to identify the mechanisms by which EPO increases FGF23 expression, as well as the mechanisms by which EPO may affect regulation of FGF23 cleavage. In the setting of elevated endogenous EPO levels or exogenous EPO administration, the degree to which increased FGF23 proteolytic cleavage offsets increased FGF23 production determines the amount of circulating bioactive intact FGF23. As such, there may be important implications in CKD, as endogenous EPO levels are increased in early CKD,[35] exogenous EPO is used almost universally in late CKD, and FGF23 cleavage may be impaired as CKD progresses.[55,56] The current study underscores the complex interrelationships among aspects of CKD-related anemia, CKD-MBD, and their respective treatment modalities.

Conflict of interest statement

The authors declare no conflicts of interest.

Author contributions

MRH, EN, IBS, and TG designed the UCLA mouse and human studies; SJLB designed the UMCG human studies; MRH, MR, KC, VG, BQ, and GJ conducted the UCLA mouse experiments; BG and GR collected the UCLA human data; SJLB and JJvZ collected the UMCG human data; MRH analyzed the UCLA mouse and human data and prepared the figures; MFE analyzed the UMCG human data and prepared the tables; MRH drafted the UCLA portion of the manuscript; MFE drafted the UMCG portion of the manuscript; MRH, MFE, MadJ, MHdB, SJLB, EN, IBS, CAJMG, and TG reviewed and revised the manuscript; all authors approved the final version of the manuscript.

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ONLINE SUPPLEMENTAL MATERIAL

Supplemental Results

Associations between serum EPO and circulating FGF23 levels in kidney transplant recipients

As presented in the main text, we assessed associations between serum EPO and circulating FGF23 levels in a large cohort of adult renal transplant recipients (RTR, n=680). We observed that Log EPO was independently and positively associated with Log cFGF23. We repeated the analyses in an independent replication cohort consisting of 592 RTRs, whose baseline characteristics are described in **Supplemental Table 3**. In this cohort, Log EPO was univariately associated with Log cFGF23 levels ($\beta = 0.32$, $p < 0.001$). In multivariable analysis, Log EPO remained a major determinant of Log cFGF23 levels, independent of adjustment for age, sex, eGFR, time since transplantation, phosphate, Log PTH, hemoglobin, and Log CRP levels ($\beta = 0.21$, $p < 0.001$). Mediation analysis showed only 14.6% mediation by hemoglobin on the association between total FGF23 and EPO, independent of potential confounders (**Supplemental Table 4**). In a sensitivity analysis, we excluded kidney transplant patients on ESA (n=13), and repeated the multivariable model. Again, Log EPO remained independently and positively associated with Log cFGF23 ($\beta = 0.22$, $p < 0.001$).

Supplemental Table 1. Baseline characteristics of UCLA non-dialysis CKD and dialysis cohort

	UCLA Non-dialysis CKD Cohort	UCLA Dialysis Cohort
Number	42	79
Sex	40% male, 60% female	70% male, 30% female
Age Group	57% pediatric, 43% adult	48% pediatric, 52% adult
Dialysis Modality	n/a	85% HD, 15% PD
Age (years)	37 ± 28	40 ± 27
eGFR (ml/min/1.73m^2)	34 ± 18	n/a
C-terminal (total) FGF23 (RU/ml)	119 (92, 205)	1648 (467, 4812)
Intact FGF23 (pg/ml)	105 (70, 165)	1054 (302, 3934)
Erythropoietin (U/l)	9.7 (7.1, 15.7)	n/a
Weekly rhEPO dose/kg	n/a	214 (116, 367)
Calcium (mg/dl)	9.1 ± 0.6	8.9 ± 0.8
Phosphate (mg/dl)	4.0 ± 1.0	5.5 ± 1.6
Parathyroid hormone (pg/ml)	97 (54, 152)	304 (157, 611)
Transferrin saturation (%)	23 ± 10	35 ± 17
Ferritin (ng/ml)	60 (31, 128)	562 (252, 895)
Hemoglobin (g/dl)	12.1 ± 1.7	12.0 ± 1.5
C-reactive protein (mg/l)	1.4 (0.6, 5.3)	3.3 (0.9, 7.4)

Data are presented as mean ± standard deviation, or median (interquartile range). CKD: chronic kidney disease, HD: hemodialysis, PD: peritoneal dialysis, eGFR: estimated glomerular filtration rate, FGF23: fibroblast growth factor 23; rhEPO: recombinant human erythropoietin.

Supplemental Table 2. Mediation analysis of hemoglobin in the association between erythropoietin and C-terminal FGF23 in 680 RTRs

Potential mediator	Outcome	Effect (path)*	Multivariable model**	
			Coefficient (95% CI)†	Proportion mediated§
Hemoglobin	**cFGF23**	Indirect effect (*ab* path)	0.02 (0.006; 0.04)	**8.3%**
		Total effect (*ab* + *c'* path)	0.23 (0.14; 0.32)	
		Unstandardized total effect	0.19 (0.14; 0.26)	

* The coefficients of the indirect *ab* path and the total *ab* + *c'* path are standardized for the standard deviations of the potential mediator, erythropoietin, and outcome.

**All coefficients are adjusted for age, sex, eGFR, time since transplantation, serum phosphate, and hs-CRP levels

§The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

†95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2000 bootstrap samples.

Supplemental Table 3. Baseline characteristics of 592 renal transplant recipients (RTRs) across tertiles of erythropoietin levels

	Tertiles of erythropoietin (IU/L)				
	All patients (n=592)	**T1 (n=197) [4.0-13.7]**	**T2 (n=199) [13.8-21.5]**	**T3 (n=196) [21.6-195.0]**	*P* value
Age (years)	51±12	48±12	52±12	55±11	<0.001
Male sex (n, %)	325 (55)	113 (57)	111 (56)	101 (52)	0.49
Body mass index, kg/m^2	26±4	26±4	26±4	27±4	0.009
Alcohol use (n, %)	295 (50)	102 (52)	94 (47)	99 (51)	0.47
Smoking status					0.24
Never smoker (n, %)	211 (36)	65 (33)	80 (40)	66 (34)	
Former smoker (n, %)	249 (42)	83 (42)	85 (43)	81 (41)	
Current smoker (n, %)	130 (22)	48 (24)	34 (17)	48 (25)	
Time since transplantation (yrs)	6.0 (2.6-11.5)	4.7 (2.2-9.3)	6.5 (3.5-11.6)	6.6 (2.7-13.8)	0.002
Diabetes mellitus (n, %)	104 (18)	37 (19)	27 (14)	40 (20)	0.18
Systolic blood pressure (mmHg)	153±23	151±21	151±21	157±25	0.02
Diastolic blood pressure (mmHg)	90±10	90±10	90±9	90±11	0.79
Laboratory measurements					
cFGF23 (RU/mL)	139 (94-218)	119 (79-169)	138 (89-204)	194 (115-356)	<0.001
Hemoglobin (mmol/L)	8.6±1.0	8.8±1.0	8.6±1.0	8.4±1.0	<0.001
MCV (fL)	91±6	90±5	91±6	92±8	<0.001
Ferritin (µg/L)	155 (76-283)	151 (82-321)	166 (98-283)	135 (64-256)	0.07
Total cholesterol (mmol/L)	5.6±1.1	5.7±0.9	5.6±1.2	5.5±1.1	0.39
Phosphate (mmol/L)	1.1±0.2	1.1±0.2	1.1±0.2	1.1±0.2	0.34
eGFR (ml/min/1.73m^2)	46±15	48±15	47±14	44±15	0.02
Creatinine (µmol/L)	147±58	145±55	143±53	154±65	0.13
Proteinuria (>0.5g) (n, %)	161 (27)	46 (23)	55 (28)	60 (31)	0.24
hs-CRP (mg/L)	2.0 (0.8-4.8)	1.5 (0.6-4.0)	1.9 (0.8-3.9)	3.0 (1.2-7.5)	<0.001
Treatment					
ACE-inhibitors (n, %)	199 (34)	83 (42)	57 (29)	59 (30)	0.008
Bèta-blocker (n, %)	365 (62)	120 (61)	127 (64)	118 (60)	0.74
Calcium channel blockers (n, %)	225 (38)	78 (40)	68 (34)	79 (40)	0.54
Diuretic use (n, %)	261 (44)	77 (39)	76 (38)	108 (55)	0.001

Values are means ± standard deviation, medians (interquartile range) or proportions (%). Abbreviations: ACE, angiotensin converting enzyme; cFGF23, C-Terminal fibroblast growth factor 23; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MCV, mean corpuscular volume.

Supplemental Table 4. Mediation analysis of hemoglobin in the association between erythropoietin and C-terminal FGF23 in replication cohort of 592 renal transplant recipients (RTRs)

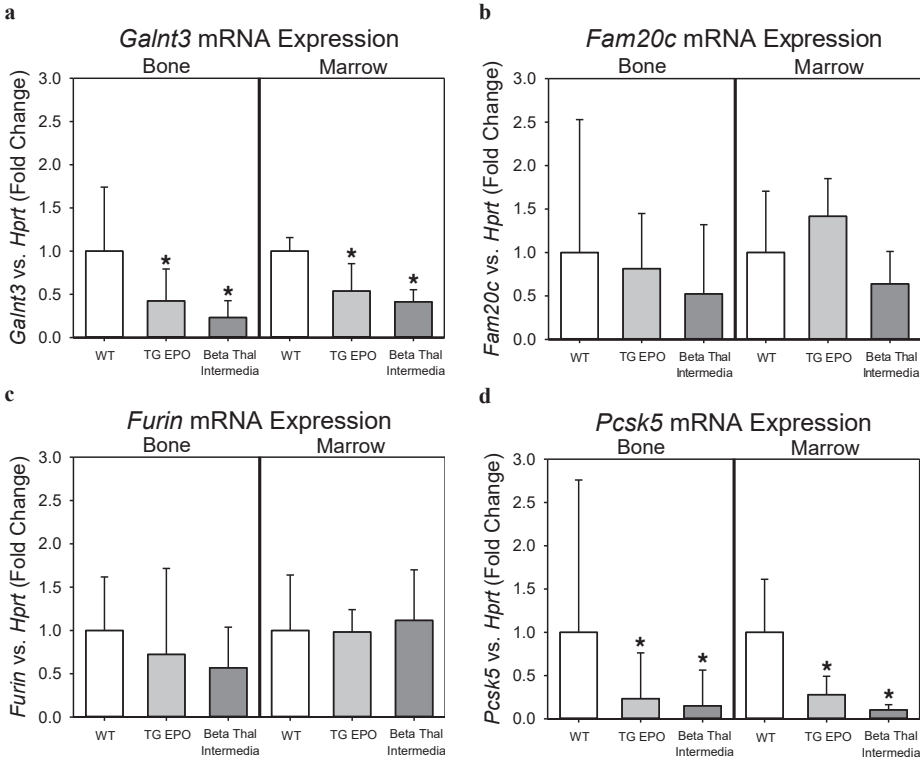
Potential mediator	Outcome	Effect (path)*	Multivariable model**	
			Coefficient (95% CI)†	Proportion mediated§
Hemoglobin	**cFGF23**	Indirect effect (*ab* path)	0.04 (0.01-0.07)	**14.6%**
		Total effect (*ab* + *c'* path)	0.25 (0.16-0.32)	
		Unstandardized total effect	0.29 (0.18-0.40)	

* The coefficients of the indirect *ab* path and the total *ab* + *c'* path are standardized for the standard deviations of the potential mediator, erythropoietin, and outcome.

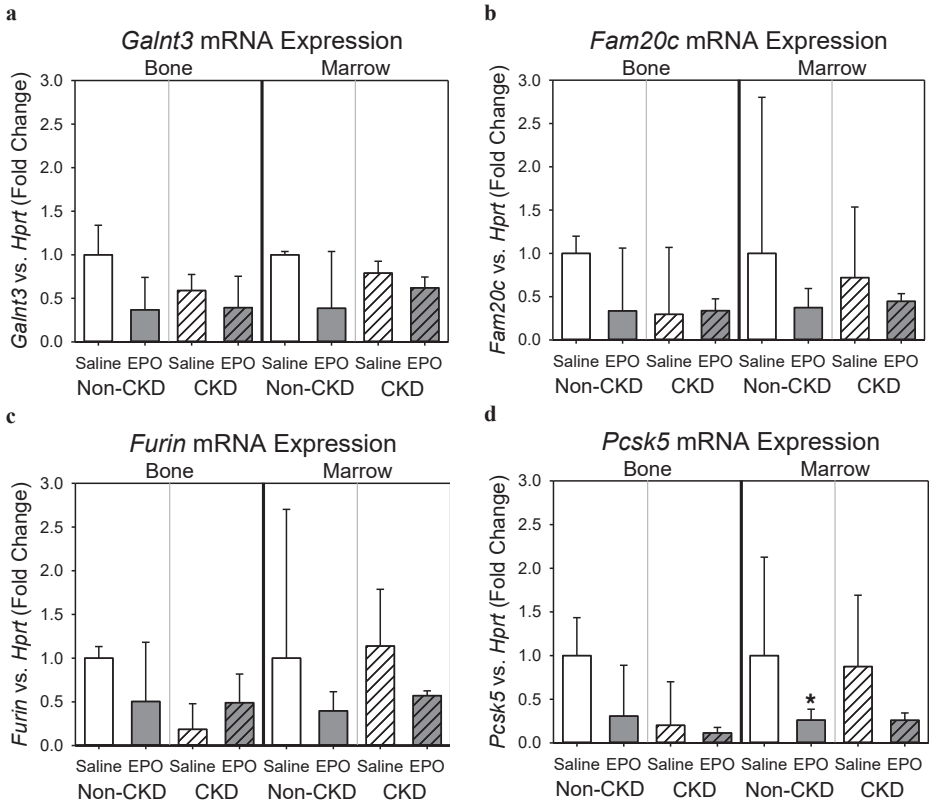
**All coefficients are adjusted for age, sex, eGFR, time since transplantation, serum phosphate, and hs-CRP levels

§The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

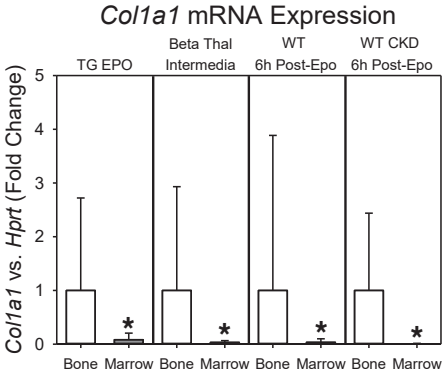
†95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2000 bootstrap samples.



Supplemental Figure 1: Bone and marrow *Galnt3*, *Fam20c*, *Furin*, and *Pcsk5* mRNA expression in high endogenous erythropoietin (EPO) models. Bone and marrow from wild type mice (WT), transgenic erythropoietin overexpressing mice (TG EPO), and beta thalassemia intermedia mice were assessed for (a) *Galnt3*, (b) *Fam20c*, (c) *Furin*, and (d) *Pcsk5* mRNA expression. * denotes a statistically significant pairwise comparison versus the WT group ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). Data are presented as means and standard deviations. n = 7-9 mice per group.



Supplemental Figure 2: Bone and marrow *Galnt3*, *Fam20c*, *Furin*, and *Pcsk5* mRNA expression in high exogenous erythropoietin (EPO) models. Bone and marrow from wild type mice with and without chronic kidney disease (CKD) were assessed for (a) *Galnt3*, (b) *Fam20c*, (c) *Furin*, and (d) *Pcsk5* mRNA expression at 6h post-EPO or saline injection. * denotes a statistically significant pairwise comparison versus the respective saline-injected group ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). Data are presented as means and standard deviations. n = 4 mice per group.



Supplemental Figure 3. Bone and marrow *Col1a1* mRNA expression. Bone and marrow from high endogenous/exogenous EPO groups (transgenic EPO, beta thalassemia intermedia, wild type mice without CKD 6h post-EPO injection, and wild type mice with CKD 6h post-EPO injection) were assessed for *Col1a1* mRNA expression, a bone marker. *p<0.05 for pairwise comparison of marrow versus bone. Data are presented as means and standard deviations. n = 4-7 mice per group.

COMPLETE METHODS

Animal studies

Mouse experiments

Experiments were conducted in accordance with UCLA Division of Laboratory Animal Medicine guidelines, and the study protocol was approved by the UCLA Office of Animal Research Oversight. Mice were housed at UCLA, in standard cages with wood chip bedding that was changed twice weekly. Animal housing rooms were temperature and humidity controlled, with a 12-hour light cycle.

Transgenic EPO-overexpressing mice

Transgenic C57BL/6 mice overexpressing human EPO (Tg6 mice)[1] were maintained on diets containing standard iron concentrations. We compared transgenic EPO mice to their wild type littermates, at the age of 7-11 weeks. A subset of transgenic EPO mice received a single intraperitoneal dose of 10 mg iron dextran (Sigma-Aldrich, St. Louis, MO) 24 hours prior to euthanasia. At the time of euthanasia, we collected whole blood, plasma, serum, livers, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Beta thalassemia intermedia mice

Beta thalassemia intermedia mice (*Hbb*$^{th3/+}$;[2] The Jackson Laboratory, Bar Harbor, ME; JAX stock #003253) were maintained on diets containing standard iron concentrations. Mice were euthanized at a median age of 17 weeks (range 9-28 weeks), and we collected whole blood, plasma, serum, livers, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Wild type mouse models, diets, and treatments

Wild type C57BL/6 mice (Jackson Laboratories) were used for experiments assessing the effects of a single rhEPO dose on FGF23. Mouse diets were obtained from Harlan Teklad (Indianapolis, IN), and contained sufficient iron (50 ppm) and standard phosphate concentrations. For groups of mice in which CKD was induced, the diets also contained 0.2% w/w adenine, as previously described.[3-7] Diets were started at 4-6 weeks of age, and provided ad libitum. Mice remained on the diets for ~5 weeks, then the experiments were conducted. Groups of mice, with and without CKD, received a single intraperitoneal dose of ~67 units/gram rhEPO (BioLegend, San Diego, CA) or saline (vehicle) and were euthanized 6 or 24 hours post-injection. Separate groups that received no injections provided baseline data. At the time of euthanasia, we collected whole blood, plasma, serum, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Mouse biochemical parameters

Complete blood counts were measured by the Hemavet® 950 automated processor (Drew Scientific, Oxford, CT). Colorimetric methods were used to assay serum urea nitrogen (BioAssay Systems, Hayward, CA), phosphate (for Tg6 and *Th3*/+ mice, Stanbio Laboratory, Boerne, TX; for wild type mice treated with EPO, Alfa-Wassermann ACE® Alera and Axcel Systems, West Caldwell, NJ), and iron (Genzyme, Cambridge, MA). Plasma human EPO (in the Tg6 mice) and serum mouse EPO (in the *Th3*/+ mice) were assayed using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). Plasma C-terminal (total) FGF23 (cFGF23) and intact FGF23 (iFGF23) concentrations were assayed using rodent-specific ELISA kits (Immutopics, San Clemente, CA and Quidel, San Diego, CA). Whereas the cFGF23 assay detects both the full-length, intact hormone and its inactive C-terminal proteolytic fragments, thus functioning as a surrogate measure of overall FGF23 production, the iFGF23 assay detects only the full-length, biologically active form. Percentage iFGF23 was calculated by dividing the iFGF23 values (measured in pg/ml) by the cFGF23 values (also measured in pg/ml) and multiplying by 100, as previously described.[7]

Quantitative hepatic iron concentration

Harvested livers were snap-frozen in liquid nitrogen and stored at -80°C. Small pieces of the livers (~100 mg) were weighed and homogenized. Protein precipitation solution (0.53N HCl and 5.3% trichloroacetic acid in ddH2O) was added, and the samples were boiled and centrifuged. Iron concentrations in the supernatants were measured by a colorimetric assay (Genzyme), then normalized to the weights of the original samples to yield liver iron concentration.

Quantitative real-time PCR

Flushed tibias and isolated bone marrow were homogenized in Trizol (Invitrogen, Life Technologies, CA) immediately after tissue collection. RNA was then isolated according to the manufacturer's protocol. We performed quantitative RT-PCR using the iScript RT-PCR kit (Bio-Rad, Hercules, CA) and primers specific for mouse *Fgf23*, *Galnt3*, *Fam20c*, *Furin*, *Pcsk5*, and *Col1a1*. Collagen 1a1 (*Col1a1*) mRNA expression was evaluated to assess for residual bone-derived cells in the marrow samples. We used the following PCR conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. Gene expression was normalized to that of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*),[8] and each RNA sample was analyzed in duplicate.

Mouse primer sequences used were:
Fgf23 forward: 5′-ACAGGAGCCATGACTCGAAG-3′;
Fgf23 reverse: 5′-GCAATTCTCTGGGCTGAAGT-3′;
Galnt3 forward: 5′-ACCAGGGAGGCAAACCATTG-3′;
Galnt3 reverse: 5′-TCCTTCTGGATGTTGTGCCG-3′;
Fam20c forward: 5′-AACCCATGAAGCAGACGAGAG-3′;
Fam20c reverse: 5′-GGAGGGACTCTGCGGAAATC-3′;
Furin forward: 5′-GCGCTCGTCCGGAAAAGTT-3′;
Furin reverse: 5′-GGACAGGGTAAGGGCCAGAT-3′;
Pcsk5 forward: 5′- ACTGCTTACACTACTACTAC-3′;
Pcsk5 reverse: 5′- GCCATATTTACAGGAGAGG-3′;
Col1a1 forward: 5-CCTCAGGGTATTGCTGGACAAC-3′;
Col1a1 reverse: 5-CAGAAGACCTTGTTTGCCAGG-3′;
Hprt forward: 5′-CTGGTTAAGCAGTACAGCCCCAA-3′;
Hprt reverse: 5′-CAGGAGGTCCTTTTCACCAGC-3′.

Statistical analysis
Statistical analysis was performed using SigmaPlot 12.5 (San Jose, CA). Mouse data are presented as means ± standard deviations.

For the mouse studies involving high endogenous EPO models presented in Figure 1, the following four t-tests were performed: (1) wild type (WT) group vs. transgenic EPO group, (2) WT group vs. transgenic EPO group treated with iron dextran, (3) WT group vs. beta thalassemia intermedia group, and (4) transgenic EPO group vs. transgenic EPO group treated with iron dextran. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

For the mouse studies involving high endogenous EPO models presented in Supplemental Figure 1, the following four t-tests were performed: (1) bone samples, WT group vs. transgenic EPO group, (2) bone samples, WT group vs. beta thalassemia intermedia group, (3) marrow samples, WT group vs. transgenic EPO group, (4) marrow samples, WT group vs. beta thalassemia intermedia group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

For the mouse studies involving rhEPO injection presented in Figure 2, within the CKD and non-CKD cohorts, the following four t-tests were performed: (1) baseline group vs. EPO-treated 6-hour time point group, (2) baseline group vs. EPO-treated 24-hour time

point group, (3) EPO-treated 6-hour time point group vs. saline-treated 6-hour time point group, and (4) EPO-treated 24-hour time point group vs. saline-treated 24-hour time point group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used. For the mouse studies involving rhEPO injection, given non-normal data distributions, plasma FGF23 levels were log-transformed prior to statistical analysis.

For the mouse studies involving rhEPO injection presented in Supplemental Figure 2, the following four t-tests were performed: (1) bone samples from non-CKD mice, EPO-treated group vs. saline-treated group, (2) bone samples from CKD mice, EPO-treated group vs. saline-treated group, (3) marrow samples from non-CKD mice, EPO-treated group vs. saline-treated group, (4) marrow samples from CKD mice, EPO-treated group vs. saline-treated group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

Human studies

UCLA human studies

The study cohort was comprised of adults and children receiving outpatient care for CKD stages 2–5 (estimated glomerular filtration rate (eGFR) <90 ml/min/1.73m^2) and 5D (dialysis-dependent). We recruited adult and pediatric non-dialysis CKD patients from UCLA general nephrology clinics. We recruited adult and pediatric dialysis patients from UCLA-affiliated Davita dialysis centers. The study was approved by the UCLA Institutional Review Board; informed consent was obtained from adult patients; and informed consent and assent were obtained from parents and pediatric patients, respectively.

Exclusion criteria were: (1) previously diagnosed non-renal cause of anemia; (2) evidence of active bleeding; (3) blood transfusion within four months of enrollment; (4) history of malignancy, end-stage liver disease, or chronic hypoxia; and (5) hospitalization or infection requiring antibiotics within four weeks of enrollment.

Following inclusion, we obtained demographic data and medication history from the medical records. Patients receiving rhEPO were on stable doses for at least four weeks prior to study enrollment, and all rhEPO administered was epoetin alfa (Epogen®, Amgen, Thousand Oaks, CA). We collected whole blood, plasma, and serum from all enrolled patients. In the dialysis patients, blood was obtained at the initiation of the hemodialysis session. This cohort was first assembled and characterized as part of a cross-sectional assessment of circulating hepcidin levels across the CKD spectrum.[9]

University Medical Center Groningen human studies

Further, we analyzed data from a cohort of post-transplant CKD patients. All kidney transplantations took place at the University Medical Center Groningen (Groningen, the Netherlands). Renal transplant recipients (RTRs) that were more than 1 year post-transplantation were approached for participation during outpatient clinic visits between 2008 and 2011, as described previously.[10] Written informed consent was obtained from 707 (87%) of the 817 initially invited RTRs. For current analyses, we excluded RTRs with missing data on EPO levels (n=27), resulting in 680 RTRs eligible for analysis.

As an independent replication cohort, we repeated the analyses in another RTR cohort with data available on EPO and cFGF23 levels. The replication cohort consisted of 606 RTRs, also all with a functional graft for more than 1 year post-transplant, which had been recruited between 2001 and 2003 at the University Medical Center Groningen. The study has been described in detail previously.[11] For current analyses, we excluded RTRs with missing data on EPO levels (n=14), resulting in 592 RTRs eligible for analysis.

Both studies have been approved by the institutional review board (METC 2008/186 and 2001/039, respectively), and adhered to the principles of the WMA declaration of Helsinki.

UCLA human biochemical parameters

Complete blood counts, including hemoglobin measurements (Sysmex automated hematology analyzer), were performed. In the serum samples, we measured the following parameters: EPO (ELISA, R&D Systems, Minneapolis, MN), creatinine (Roche cobas® 8000 analyzer enzymatic assay, Switzerland), phosphate (Roche cobas® 8000 analyzer ammonium phosphomolybdate photometric assay, Switzerland), calcium (Roche cobas® 8000 analyzer 5-nitro-5′-methyl-BAPTA (NM-BAPTA) photometric assay, Switzerland), intact parathyroid hormone (PTH; first generation Immutopics assay, San Clemente, CA), iron and total iron binding capacity (Roche cobas® 8000 analyzer Ferrozine colorimetric assay, Switzerland), ferritin (Roche cobas® 8000 analyzer electrochemiluminescence assay, Switzerland), and high sensitivity C-reactive protein (CRP; CardioPhase® hsCRP assay, Dade Behring, Deerfield, IL). Transferrin saturation (TSAT, %) was calculated by iron divided by total iron binding capacity. In the plasma samples, we measured total FGF23 (cFGF23; Immutopics/Quidel) and intact FGF23 (iFGF23; Immutopics/Quidel) concentrations. In the non-dialysis CKD patients, we also measured iFGF23 levels using both the Kainos Laboratories (Tokyo, Japan) assay. In adult patients, eGFR was calculated with the Modification of Diet in Renal Disease (MDRD) Study equation.[12] In pediatric patients, eGFR was calculated with the revised Schwartz equation.[13]

University Medical Center Groningen human biochemical parameters

Blood was drawn in the morning after an 8-12h overnight fast. Total (cFGF23) levels were measured in stored plasma samples using the human FGF23 (C-terminal) ELISA

(Immutopics/Quidel). Intact FGF23 levels were measured in stored plasma samples by ELISA (Kainos Laboratories). cFGF23 data were available in both cohorts, but iFGF23 data were available only in the larger cohort of 680 RTRs. In both studies, EPO levels were measured using an immunoassay based on chemiluminescence (Immulite EPO assay, Los Angeles, CA). Further, we measured transferrin (Cobas c analyzer, Modular P system, Roche diagnostics, Mannheim, Germany), ferritin (Modular analytics E170, Roche diagnostics), and serum iron (Modular P800 system; Roche diagnostics). Transferrin saturation (%) was calculated as 100 x serum iron (µmol/L) / 25 x transferrin (g/L). Renal function was determined by estimating GFR by applying the Modification of Diet in Renal Disease (MDRD) Study equation.[12]

UCLA statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (San Jose, CA). Normally distributed data are presented as means ± standard deviation, skewed distributed data are presented as medians with interquartile range, and categorical data are presented as numbers (percentage). Multiple linear regression models, adjusted for demographic, anemia-related, and mineral metabolism covariables (age, eGFR, calcium, phosphate, PTH, TSAT, ferritin, hemoglobin, and CRP), were developed to investigate the association between serum EPO levels and FGF23 in the non-dialysis CKD cohort, and between rhEPO dose and FGF23 in the dialysis cohort.

University Medical Center Groningen statistical analysis

Statistical analysis was performed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL) and STATA 14.1 (STATA Corp., College Station, TX). Normally distributed data are presented as means ± standard deviation, skewed distributed data are presented as medians with interquartile range, and categorical data are presented as numbers (percentage). Differences in baseline characteristics across EPO tertiles were evaluated with one-way ANOVA, Kruskal-Wallis test, or Chi-square test, as appropriate. Linear regression analysis was performed to assess whether EPO is a major determinant of cFGF23 or iFGF23 levels after adjustment for age, sex, eGFR, time since transplantation, calcium, phosphate, PTH, hemoglobin, ferritin, and CRP levels. In addition, we performed multivariable stepwise backward linear regression analysis to determine whether EPO remained a determinant of cFGF23 levels alongside known determinants. Finally, we determined in mediation analysis according to Preacher and Hayes,[14,15] based on logistic regression, whether hemoglobin mediated the association between EPO and cFGF23 independent of adjustment for potential confounders. In all analyses, a two-sided p-value <0.05 was considered significant.

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